Enhancing Malaria Vaccine Development by the Naval Medical Research Center

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14. ABSTRACT

(Report developed under SBIR contract for topic OSD00-HP-02.) A priority of DOD is to develop effective vaccines for preventing malaria. Developing malaria vaccines is complicated by the complexity of the parasite and of the human host's response to the infection. Developing sustainably effective vaccines requires a vaccine delivery system that can accommodate a complex variety of biologicals targeted for immune stimulation.

In this project, DNA plasmid-based vaccines were encapsulated within biodegradable polymer microparticles using proprietary techniques. The delivery vehicle was designed to protect the pDNA-based vaccine from degradation and provide a sustained release of the vaccine over time. In vitro approximately 60 to 80 percent of the plasmid was released within 24 hours. The plasmid was then released at a controlled rate close to 1 percent per day for 21 days. The total amount of plasmid released after 21 days ranged between 70 and 90 percent.

In vivo, mice received a subcutaneous injection of particles. Although there were no detectable antibody or cytotoxic T lymphocyte responses two weeks post-immunization, previous data support the fact that optimal immune responses occur when vaccines are administrated intramuscularly. Additional studies will be undertaken in Phase II to look at intramuscular injection of pDNA/polymer-based vaccines in parallel with naked pDNA controls to establish efficacy of vaccines encapsulated within the polymer particles.

15. SUBJECT TERMS

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1.0 PHASE I PROJECT SUMMARY

Plasmid-based vaccines were encapsulated within biodegradable polymer microparticles to produce a novel delivery system for immunizations. Vaccines designed to induce antibodies specific for antigens found on the surface of malaria-infected hepatocyte cells were received from the Naval Medical Research Center. The polymeric delivery vehicle was designed to protect the pDNA-based vaccine from degradation *in vivo* and provide a sustained release of the vaccine with respect to time. Vaccines were loaded within porous polymer matrices, compressed into a pellet to encapsulate the pDNA, and cryogenically ground to produce microparticles. Microparticles with an average size of 15 μm were suspended in phosphate buffer and release of pDNA from the polymer was monitored *in vitro*. Approximately 60 to 80 percent of the plasmid was released within 24 hours after exposure to the aqueous buffer. Following the initial burst, pDNA was released at a controlled rate close to an additional 1 percent per day for 21 days. The total amount of plasmid released after 21 days ranged between 70 and 90 percent for all of the vaccines under study. No detectable plasmid concentration was released from the particles after 21 days.

The ability of the pDNA/polymer-based vaccines to induce antibody response was tested in a mouse model. Mice received a subcutaneous injection of particles suspended in an aqueous solution at weeks 0 and 3. There was no detectable antibody or cytotoxic T-lymphocyte response in mice following inspection two weeks post the second immunization. Previous studies indicated that optimal immune response occurred in vaccines administrated via intramuscular injections. Additional studies were proposed for Phase II to observe intramuscular injection of pDNA/polymer-based vaccines in parallel with naked pDNA (pDNA in saline) controls to establish efficacy and dose response of vaccines encapsulated within the polymer particles.

2. BACKGROUND AND SIGNIFICANCE

2.1 Malaria Vaccine Development

Each year approximately 300 to 500 million people are infected with malaria and each year 1.5 to 2.7 million people die from this disease (Gardner et al., 1998; WHO, 1997). Since World War II, the struggle against malaria has gone through several stages. The first stage involved a massive effort aimed at eradicating the vector. The second stage was the development of antimalarial drugs based on quinine derivatives and alternatives (Shulemann, 1932; Corell et al., 1955).

Due to introduced drug resistance, Miller and Hoffman (1998) stated that vaccination represents the best potential for control of the disease. The third stage of malaria control, then, recognizes the limitations of vector control and chemotherapy. In this regard, a current emphasis is on development of DNA-based vaccines against one or more of the developmental forms of the malaria parasite. Various strategies have been explored for implementing DNA-based vaccines.

Vaccines may prove beneficial to a wide range of populations. Proposed goals aim to prevent disease in foreign travelers and residents in low transmission areas such as India and reduce disease in high transmission areas such as sub-Saharan Africa. Even vaccines demonstrated to provoke only low levels of antibodies might be useful in priming the immune system. Subsequent natural infection would help reduce the disease in high-risk populations such as children and pregnant women of Africa (Hoffman et al., 1998). The potential and applicability of malaria vaccines as a treatment method has led to the development of a number of candidates. Several additional candidate vaccines are expected in coming years upon sequencing of the *P. falciparum* genome (Gardner et al., 1998).

Vaccine trials have progressed from mice (Doolan et al., 1996) to monkeys (Wang et al., 1998) and into humans (Stoute et al., 1997; Wang et al., 1998). Malaria vaccines work by inducing the production of $CD8^+$ T-cells that kill infected hepatocytes. Immunity stems from recognition of peptides present on the surface of infected hepatocytes by $CD8^+$ T-cells that mediate infected cell elimination. Doolan et al., (1996) demonstrated partial protection ranging from 8 to 75 percent among various breeds of mice inoculated intramuscularly with DNA encoding for the Plasmodium yoelii circumsporozoite protein (PyCSP). Protection ranging from 80 to 90 percent was conferred onto mice by injection of a combination of plasmid vaccines namely PyCSP and Plasmodium yoelli hepatocyte erythrocyte protein 17 (PyHEP17). The success of the combination was attributed to a circumvention of genetic restrictions that lessened protective immunity mediated by $CD8^+$ T-cells. Clinical vaccines are likely to include several protein-inducing plasmids to overcome genetic restrictions and handle parasite polymorphism.

Wang et al., (1998) applied the concept of multigene immunization in a study involving rhesus monkeys. Monkeys were injected three times at weeks 0, 4 and 16 at each of four intramuscular sites. The induction of antigen-specific antibodies required multiple immunizations. No antibodies were detected in any of the subjects following the first immunization. However, 8 of 12 monkeys demonstrated antibody production following the second immunization and 11 of 12 after the third immunization. Furthermore, 8 of 12 animals expressed $CD8^+$ T-cell responses to all of the delivered epitopes and three additional animals showed $CD8^+$ T-cell responses to all but one. These results help to support the effectiveness of the multiple epitope immunization approach.

Based on the encouraging results in nonhuman primates, Hoffman et al., (1997) proposed a plan to clinically test a multigene malaria vaccine in humans. Twenty malaria naïve volunteers were given three immunizations of the *P. falciparum* liver-stage DNA vaccine. The induction of $CD8^+$ T-cell against the expressed protein was monitored by collection of peripheal blood mononuclear cells. Wang et al., (1998) reported that immune responses were detected in doses as small as $20\mu g$, but doses ranging from 500 to $2500\mu g$ elicited responses to approximately 70 percent of all of the peptides studied. In general, the magnitude of the immune response was also reported to be significantly higher than observed in humans exposed to conventional irradiated sporozoites or natural infection alone. Le et al., (2000) conducted safety studies and subjects observed mostly mild systems through one year following immunizations. However, the effectiveness of the vaccine was questioned, as there were no detectable antigen-specific antibodies present despite an induction of $CD8^+$ T-cell response. Stoute et al., (1997) conducted independent clinical trials of *P. falciparum* vaccines with mixed results. Human volunteers were vaccinated and then exposed to infection causing development of malaria in 100 percent of control subjects. Two vaccine formulations had little effect as the majority of volunteers contracted the disease, but a third formulation prevented malaria in seven of eight volunteers. Further studies were indicated to determine vaccine safety and reasons why the third formulation may have been more successful than others.

There have been measures to improve vaccine efficacy. Sedegah et al., (1998) demonstrated increases in protection by priming with the malaria vaccine and boosting with recombinant vaccinia. Malaria challenged mice demonstrated protection in 69 percent of the subjects boosted with vaccinia PyCSP, versus 44 percent of animals immunized with the DNA vaccine alone. Subsequent studies by Sedegah et al., (2000) combined both boosting with vaccinia PyCSP and coadministration of a plasmid expressing murine GM-CSF. Priming with PyCSP plasmid DNA and plasmid GM-CSF was demonstrated to confer protection to 100 percent of challenged mice dependent upon amount of recombinant vaccinia delivered during boosting.

2.2 Use of PLGA in Vaccine Development: Delivery of Proteins and Peptides

The use of homo- and copolymers of lactide and glycolide for biomedical applications is well-established and is based on the biocompatibility of these materials and their degradation products, lactic and glycolic acids (Visscher et al., 1985). Rates of degradation and release of incorporated active agents are dependent both on the molecular weight of the polymer and on the lactide-to-glycolide ratio. A sample list of proteins and peptides, which have been incorporated into this PLG of polymers, has been compiled by Cleland and Langer (1994).

Traditional emulsion techniques for PLGA vaccines use blenders to generate the emulsions. However, the energy of this process results in some degradation of the DNA. As a consequence a large portion of the supercoiled material was degraded to the open circle or linear form. The damage is a consequence of the shear forces acting on the liquid components of the emulsion. The patented process proposed here does not involve emulsion formation and can be referred to as a "solid state" technique; this process should result in limited damage to the biological. Particle size reduction is accomplished by low temperature grinding (–40° to –50°C) of solid particles in which shear forces on liquid droplets do not occur. The impact on solid particles transfers energy to the particle that dissipates on fracture and results in only a transient temperature rise. Thus, denaturation or other destructive processes are limited.

2.3 Delivery of Plasmid-Based Vaccines

The effectiveness and longevity of pDNA vaccines may be improved by incorporation of pDNA within polymeric delivery vehicles. Administration of naked pDNA leaves the vaccine vulnerable to attack from degradation enzymes that can reduce half-lives to minutes or hours (Kawabata et al., 1995, Luo and Saltzman 2000). Chemical modification of DNA has previously been utilized to protect the vaccine from nucleases and increase vaccine longevity (Benns and Kim 2000, Luo and Saltzman 2000). Modified vaccines have been complexed with cationic and anionic liposomes, polysaccharides, poly(ethylene glycol), and poly(L-lysine) among others. A drawback to chemical modification has been increases in systemic toxicity resulting from exposure to the complexed chemicals (Luo and Saltzman 2000).

A second alternative involves encapsulation of the plasmid within a polymeric carrier. Biodegradable homo- and copolymers of lactide and glycolide (the "PLGAs") provide protection for the plasmid, while enabling a sustained and controlled release of the plasmid. Anchordoquy and Koe (2000) reviewed the stability of plasmid-based therapies and suggested that polymeric carrier vehicles such as copolymers of lactide and glycolide (PLGA) may have potential to isolate and entrap DNA. Isolation of the plasmid may prove to be beneficial in reducing negative interactions such as aggregation that leads to loss of biological activity in typical liquid formulations. The application of PLGA to biomedical applications is well established and is based on the biocompatibility of these materials and their degradation products, lactic and glycolic acids (Visscher et al., 1985). Control of plasmid release may improve vaccine efficacy because prolonged availability may enable sustained gene expression (Labhasetwar et al., 1998).

Recent researchers have studied the encapsulation of plasmid-based therapeutics within polymer-based vehicles. Tinsley-Bown et al., (2000) demonstrated the release of a firefly luciferase-derived plasmid from microcapsules of a PLGA. *In vitro* studies found that the release rate of the plasmid into solution was dependent upon polymer molecular weight. Perez et al., (2001) encapsulated plasmid DNA into nanoparticles of poly(lactic acid) and poly(ethylene

glycol) copolymers. In this study, plasmid loadings of 10 - 12 μg per mg of polymer resulted in a large initial burst of plasmid from the matrix followed by a slower release for 28 days.

Whereas polymeric carriers provide advantages over naked pDNA injections, loss of vaccine effectiveness in terms of physical mass loss and structural rearrangement of pDNA has been observed for encapsulation within polymeric delivery vehicles. Encapsulation efficiency of pDNA within PLGA matrices has varied with technique. Various procedures modified from the traditional double emulsion/solvent evaporation technique have yielded encapsulation efficiencies in the range of 20-50 percent (Tinsley-Brown et al., 2000) and 30–35 percent (Capan et al., 1999). However, Cohen et al., (2000) reported a higher efficiency, 70 percent, for encapsulation of pDNA within nanoparticles of PLGA than otherwise found. In addition to mass loss during the encapsulation procedure, rearrangements of pDNA structure have also been reported. A significant decrease in the percentage of supercoiled pDNA in favor of open circle pDNA has been reported. Tinsley-Brown (2000) reported that 30–40 percent of pDNA was recovered in the supercoiled form with losses being attributed to the open circle conformation. Capan et al., (1999) observed an increased loss of supercoiling, 16 percent, for uncomplexed pDNA. However, through forming of pDNA-poly(L-lysine) complexes, the percentage of pDNA remaining in the supercoiled structure increased to 75–85 percent.

The effectiveness of pDNA vaccines delivered in a PLGA vehicle has been demonstrated *in vivo*. Cohen et al., (2000) showed that a sustained release of pDNA from PLGA microparticles increased expression of alkaline phosphatase versus an injection of naked pDNA beyond 7 days. However, injections of polymer-encapsulated pDNA resulted in less expression versus naked pDNA for a period of 72 hours post-injection. This observation was attributed to the reduced availability of encapsulated pDNA with respect to the naked pDNA solution or diminished effectiveness of the vaccine due to rearrangements of pDNA structure. Yet, the polymeric delivery vehicle enables sustained release of pDNA vaccine. Lunsford et al., (2000) demonstrated persistence of pDNA within specific tissues in mice for a period of 120 days following injection for intramuscularly or subcutaneous injections. Tissues exposed to injections of naked pDNA were observed to be absent of pDNA beyond 15 days post-injection. Vaccine effectiveness may also be benefited by the potential of the polymeric particles to mediate transfection of macrophages during phagocytosis (Cohen et al., 2000).

2.4 Materials and Methods

Plasmids

Vaccines were received as a frozen solution of plasmid in phosphate buffered saline (PBS) from the Naval Medical Research Center, Malaria Research Program (Silver Spring, MD). Vaccines were kept frozen at –20°C prior to use. The plasmids under study were VR1020, a 5048 bp control plasmid. Vaccines were created by insertion of DNA into VR1020 plasmids. A native plasmid (VR2516) and a synthetic plasmid (VR2578) encoded for the *Plasmodium yoelii* circumsporozoite protein (PyCSP). In addition, native (VR2515) and synthetic (VR2579) plasmids were provided that encoded for the *Plasmodium yoelii* hepatocyte erythrocyte protein (PyHEP17). The synthetic plasmids were designed for optimal expression in mammalian systems. A final plasmid (VR2533) was used that encoded for PyMSP1. Plasmid sizes ranged from 5000 bp for the control plasmid to between 6,000 and 11,000 bp for the vaccines.

Particle Preparation

PLGA with a lactide to glycolide ratio of 75:25 (RG 752, Boehringer-Ingelheim) and a molecular weight of approximately 12,000 was dissolved in glacial acetic acid (Fisher Scientific, Fairlawn, NJ) at a polymer concentration of 50 mg/mL. The polymer solution was quick-frozen and then dried 8 to 12 hours by lyophilization on a Labconco model Freeze Dryer to produce a porous PLGA matrix. A dried foam density of 0.68 mg/mL was calculated by measuring the volume of water displaced by a section of the porous material. Based upon a nonporous polymer density of approximately 1.2, the void fraction of the polymer matrix was calculated to be 94 percent.

Impregnation of the porous PLGA matrices with plasmid was achieved by loading a diluted pDNA solution into the pores. The volume of solution added to the porous polymer was equal to 110 percent of the calculated void volume. Excess solution ensured that voids within the polymer would be saturated. Concentrated pDNA solutions were diluted in distilled water and the plasmid solution was added to the polymer matrix. Incorporation of the plasmid was achieved by slowly depressuring the polymer and pDNA solution to immerse the porous matrix within the solution. Saturation of the polymer matrix was determined by the disappearance of free solution as the pores of the matrix were filled. The quantity of pDNA in solution amounted to 1 percent of the total polymer mass or 10 μg pDNA per mg PLGA.

Following depressurization of the mixture and saturation of the polymer matrix, the foam was quick-frozen. Water was removed from the polymer and pDNA by lyophilization for 48 hours. The dried polymer matrix was then compressed at 20,000 psi for 2 h in order to impregnate the precipitated pDNA within a PLGA pellet. Following compression, the polymer pellet was ground cryogenically at –5°C for 5–10 min in order to form a powder. Particle size of the PLGA powder was estimated by observing a sampling of the PLGA particles via light microscopy. Particle sizes ranged from 15-50 μm with an average diameter of approximately 25 μm.

Encapsulation of pDNA

The concentration of encapsulated pDNA was measured by extracting pDNA from the polymer matrix. The extraction procedure involved removal of the pDNA by promoting rapid release from the polymer matrix (Tinsley-Brown et al. 2000). Approximately 10 mg of the PLGA particles with encapsulated pDNA were added to 1 mL of 0.2M NaOH. The suspension was vigorously mixed on a Fisher Scientific vortexor, model G-560, for 1 min. The polymer and NaOH solution was then incubated at 120°C for an additional 10 min. Following incubation, particles and solution were isolated by centrifugation at 5000 g for 10 min. The supernatant containing the released pDNA was removed from the polymer with the aid of a pipette. The concentration of released pDNA in NaOH solution was determined by UV spectroscopy.

In Vitro Release Studies

In vitro release studies of pDNA from the PLGA particles were conducted to measure the release rate of plasmid from the matrix. PLGA particles containing the encapsulated pDNA with an approximate mass of 10 mg were suspended in 1 mL of 0.1 M phosphate buffer saline (PBS). The suspension was incubated at 37°C and shaken at 45 cycles per minute in a PolyScience model 28L water bath. A total of 30 samples were added to the water bath and three samples were removed at times of 1, 2, 3, 4, 7, 14, 21, 28, 35, and 42 days. Upon removal from the water bath, suspended particles were isolated by centrifugation at 8500 g for 10 min. The supernatant

solution containing released pDNA was collected with a pipette. Concentration of pDNA in PBS was determined by UV spectroscopy.

Concentration of pDNA

The concentration of pDNA in solution was measured by UV spectroscopy as described by Tinsley-Brown et al. (2000). Approximately 0.5 mL of pDNA solution was added to a quartz cuvette of path length 1 cm and width of 0.2 cm. Solutions of the original pDNA that was not encapsulated into PLGA were diluted in PBS to known concentrations. These solutions with known concentrations of pDNA were used to measure the unknown concentrations of pDNA by creating an absorbance versus concentration standard curve. Absorbance was recorded at 260 nm for each solution on a Varian Cary Scan 100 UV/Vis spectrophotometer. A reference absorbance background was provided by a PBS solution that was incubated with control PLGA particles not encapsulated with pDNA. For concentration measurement in NaOH, solutions of known pDNA concentrations were created in 0.2 M NaOH for the calibration curve. In addition, the reference background was a NaOH solution incubated with control PLGA particles that did not contain any plasmid.

In addition to UV spectroscopy, an alternative method of measuring plasmid concentration was explored using microfluidic technology. The Bioanalyzer 2100 and DNA 7500 or 12000 LabChip™ kits (Agilent Technologies, CA) were used to size and quantify plasmid DNA. The technique used microfluidics technology for DNA separation in a linear polymer gel analogous to agarose. As the sample passed through 50 to100 μm channels, DNA fragments adsorbed to a fluorescent dye that were subsequently read by a laser detector in the range of 635-638 nm. The size and quantity of the DNA fragments were determined by comparison to known markers. The Bioanalyzer was connected to a computer loaded with dedicated software in order to monitor the separation and generate a digital image.

Plasmid fragments were created from pDNA released from PLGA particles. The pDNA solutions in PBS were incubated with a restriction enzyme (Hind III) for 1 hour at 37°C. Fragments were immediately loaded into the chip and separated within channels of the LabChip™ over a 45 min. period. A total sample size of 10 μL was loaded into the chip for each plasmid. The migration time of DNA fragments within the chip was used to determine size (in base pairs) of the DNA fragments and area under the peaks was used to calculate concentration.

The protocol for analyzing DNA loaded into the LabChip™ involved several steps, carried out at room temperature with reagents already equilibrated at room temperature. First, a gel-dye mix was prepared and loaded into the wells marked with a black-colored "G", by applying pressure with a syringe. Then the marker mix was added to the ladder well and to the 12 sample wells, and 1 ml of DNA ladder is added to the ladder well. Finally, 1 ml of each digest sample was placed into the sample wells in triplicate. Samples were diluted as needed to bring the DNA concentration in the range of 0.5–5 ng/ml. One ml sample buffer was added to unused wells. The chip was shaken on a vortex device equipped with a dedicated adaptor, for one minute at medium speed (<0.5). The chip was then placed into the Bioanalyzer within 5 min of preparation for analysis. After analysis, water was placed on a cleaning chip, which serves to clean the electrodes for 30 s.

Dose Response

The dose responses of the malaria vaccines were studied in a mouse model at NMRC. BALB/c mice were injected with 100 μL of either naked VR2515 in saline solution or VR2515 encapsulated within PLGA micropartices suspended in a carboxy methyl cellulose solution. The medium used to suspend the PLGA particles prior to injection consisted of 0.36 percent (w/v) sodium carboxy methyl cellulose, 3.6 percent (w/v) D-mannitol, and 0.07 percent (w/v) Tween 80 in distilled water. Mice were immunized twice with the second round of injections occurring three weeks following the first immunization. A total of six mice received the control immunization consisting of 50 μg VR2515 in 100 μL saline injected into the tibialis muscle. Delivery of the plasmid encapsulated in PLGA was achieved by a subcutaneous injection of the particle suspension. A total of six animals were injected at each particle concentration ranging from 0.05, 0.5 or 5 mg PLGA per 100 μL of the suspension medium. With a plasmid concentration of approximately 10 μg per mg PLGA, these immunizations corresponded to doses of 0.5, 5, or 50 μg plasmid per animal.

The immune response of mice vaccinated with plasmid/PLGA vaccines was determined. At two weeks post the second immunization, animals were bled 200 to 300 μL from the tail vein with the blood to be tested for the presence of antibodies. An indirect fluorescent Ab test (IFAT) was used to detect serum levels of anti-*Plasmodium-yoelii* sporozoite antibodies. Briefly, collected sera were incubated with air-dried sporozoites and antibody concentration was measured through binding of fluorescein isothiocyanate-labeled anti-mouse Ig as described by Sedegah et al. (1998).

Protective immunity of mice immunized with the pDNA/PLGA vaccines versus mice immunized with naked pDNA was determined via cytotoxic T lymphocyte (CTL) and gamma interferon (IFN-γ) responses. CTL activity was studied using a ^{51}Cr release assay conducted on spleen cells harvested from immunized mice. Spleen cells were incubated *in vitro* with ^{51}Cr-labeled target cells containing the epitope of interest. The net percent specific lysis was calculated based upon the percent lysis of positive target cells minus the percent lysis of negative controls (Sedegah et al. 2000). IFN-γ response was measured by incubation of spleen cells with target cells containing the epitope of interest. Levels of IFN-γ were found by the addition of anti-mouse IFN-γ antibody. The numbers of IFN-γ -spot forming cells are counted per million spleen cells (Sedegah et al. 1998). Protective immunity is established by demonstration of both CTL and IFN-γ activity.

3. RESULTS AND DISCUSSION

Vaccine Encapsulation

Each plasmid vaccine was encapsulated individually into PLGA particles as described. Encapsulation efficiency and *in vitro* release of each pDNA vaccine from the polymeric matrix was studied. Although this study used single vaccine formulations, similar procedures would be applicable towards the production of multigene delivery vehicles.

A total of five plasmid vaccines and an additional control plasmid were loaded individually into PLGA particles. The theoretical loading for each plasmid was 1 percent by mass (10 μg per mg) in the polymer matrix. Plasmid loading concentrations typically ranged between 9-11 μg plasmid per mg PLGA as summarized below.

Plasmid	Concentration (μg/mg)
VR2515	10.9 ± 1.8
VR2516	10.2 ± 1.6
VR2533	6.2 ± 0.8
VR2578	8.3 ± 0.4
VR2579	10.2 ± 0.6
VR1020	16.0 ± 4.0

The encapsulation efficiency was approximately 100 percent for all of the vaccine formulations except VR2533 where only 60 percent of the pDNA was recovered. The relatively high concentration of VR1020 was attributed to scatter of the data due to a relatively small sample size available. Recovery of solid particles during the grinding procedure was approximately 90 percent of the total mass used for all of the samples. The encapsulation procedure was demonstrated herein to be an efficient method to encapsulate the vaccine and minimize loss.

In vitro Vaccine Release

Plasmid release from the polymer particles occurred at a rapid rate as expected. Although grinding created fine particulates necessary for injection, the production of microparticles resulted in a high surface area. Therefore, a significant portion of the plasmid was immediately exposed to the buffer at the polymer surface. Presence of plasmid not encapsulated within the polymer but at the surface, resulted in a large initial burst or high concentration of solubilized pDNA at the earliest time points. In addition, due to the relatively small plasmid size (<11,000 bp) diffusion of the plasmid through the polymer matrix occurred rapidly. Plasmid encapsulated within the polymer was released relatively rapidly due to the ability of the pDNA to diffuse through the polymeric matrix. Even though a majority of the plasmid is released at relatively short times, detectable increases in plasmid concentration were measured up to 21 days *in vitro*. The PLGA particle vehicle demonstrated the capability to deliver model malaria vaccines at a release rate that extended for a period of 21 days.

Release of vaccines from the polymer matrix was monitored with respect to time. Results are summarized in Figure 1 for the control plasmid (VR1020) and a vaccine plasmid (VR2516). The majority of plasmid was released immediately upon exposure of the particles to buffer. After one day, 59.6 percent of the vaccine, VR2516, was released from the polymeric matrix into the buffer. An additional 5 percent of the plasmid was released between Days 1 and 21, with most of the release occurring within the first week. The control plasmid, VR1020, was released at a similar rate between Days 1 and 21, but a larger percentage, 71.0, was present in the buffer after the first day. The release profile observed for both plasmids were typical for the PLGA system under study. A large quantity of the encapsulated material was immediately released upon exposure to buffer followed by a slower and more controlled release after Day 1. There were no detectable increases in the concentration of plasmid in the buffer beyond Day 21.

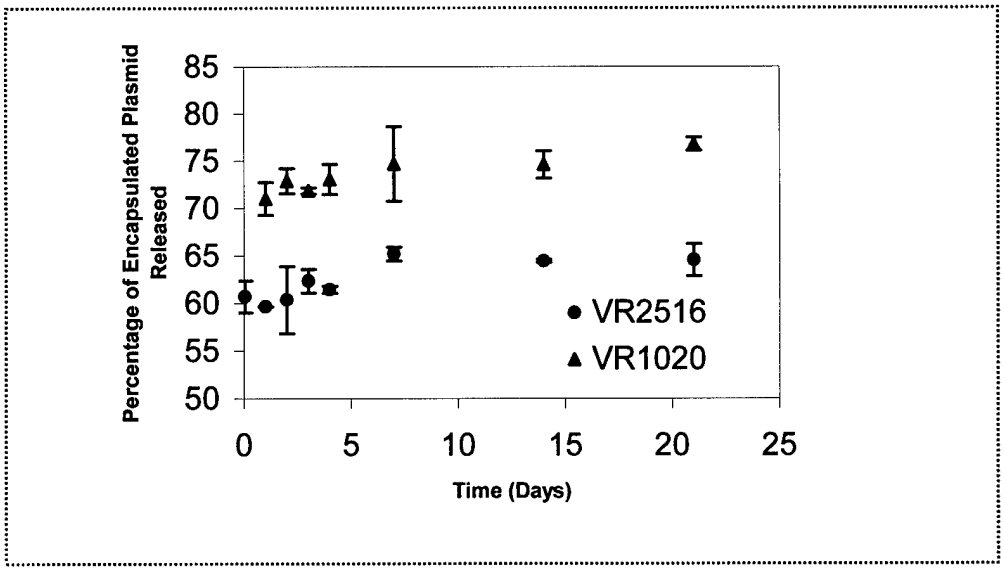

Figure 1. ***In vitro*** **release of plasmid from PLGA microparticles. Percentage refers to the amount of plasmids released from the matrix versus the total amount of plasmid loaded into the matrix.**

The concentration of plasmid released from PLGA microparticles was also studied using the Bioanalyzer 2100. Potential advantages of this procedure include smaller samples sizes and improved precision. Separation on the chip required 8 μL of sample whereas UV spectroscopy used between 0.5 mL and 1 mL. The decrease in sample volume was beneficial due to the relatively small amount of plasmid available for testing. Furthermore, precision of the concentration measurement improved as detection limits on the order of 0.5 to 5 ng/mL were possible using the Bioanalyzer compared to values of 5 to 100 μL/mL possible with UV spectroscopy.

The capability of the Bioanalyzer to separate the pDNA of the vaccines tested was studied. The efficiency of pDNA digestion was analyzed for two different concentrations of the Hind III restriction enzyme. Digested plasmids were separated on the chip with results shown in Figure 2. Columns and bands are analogous to results obtained using gel electrophoresis. Columns or wells 1, 2, and 3 were produced using a Hind III concentration of 20 units per well whereas wells 4, 5, and 6 contained Hind III at a concentration of 100 units per well. The upper and lower markers for all samples were present at the same site as that in the ladder, as expected. However, the intensity of the band corresponding to the vector in well 1 was not as great as that in wells 2, 3, 4, 5, and 6. The intensity of bands in wells 2, 3, 4, 5, and 6 were similar and no other distinctive bands were present. Wells 7, 8, 9, 10, 11, and 12 were empty and only contained the upper marker, lower marker, and gel.

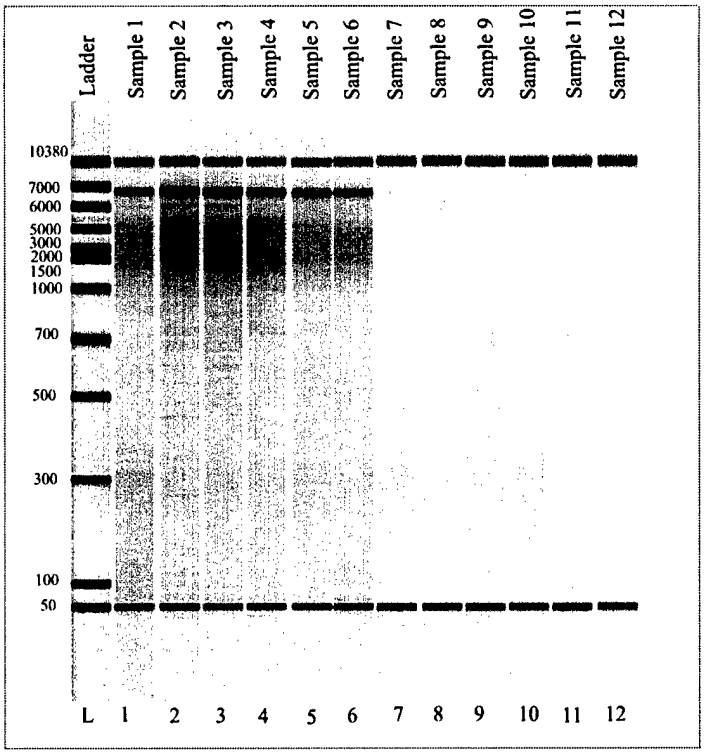

Figure 2. Separation pDNA fragments as a function of size in base pairs obtained on the Bioanalyzer 2100. Columns 1,2, and 3 consisted of pDNA and 20 units of Hind III restriction enzyme, whereas columns 4, 5, and 6 contained pDNA and 100 units of Hind III. Upper (10,380 bp) and lower (50 bp) bands were used as markers, whereas the middle band corresponded to VR2516 at approximately 6200 bp.

In this experiment, both 20 and 100 units of Hind III yielded a linear vector detectable by the Bioanalyzer. Both amounts of restriction enzyme were sufficient to digest the pDNA, as the plasmid was linearized and fully detected by the Bioanalyzer. The relatively lower intensity of the band in well 1 in comparison to the other wells was attributed to a lower concentration of the plasmid in solution. There was only one peak in addition to the lower and upper marker peaks after digestion of the vector because the gel only contained the linearized vector, an upper marker, and a lower marker. Therefore, there should be no significant peaks other than at the size (bp) of the vector.

Preliminary experiments conducted on the Bioanalyzer indicated that the procedure could be used to separate DNA fragments and quantify the size and concentration of plasmid in solution. Figure 3 represents the fluorescence spectra obtained versus migration time of the DNA fragments digested with 100 units of Hind III per well. As shown in Figure 3, the results yielded nice, clean peaks for triplicate samples of vector VR2516. The first and last peaks (green and red, respectively) are the lower and upper markers, and the middle peak (blue) is the pDNA. This graph shows that the reproducibility of the assay is good especially with respect to identical upper and lower markers and the same patterns for the pDNA. Because the pDNA peaks were easily resolved, the method would be appropriate for measurement of plasmid concentration during future studies.

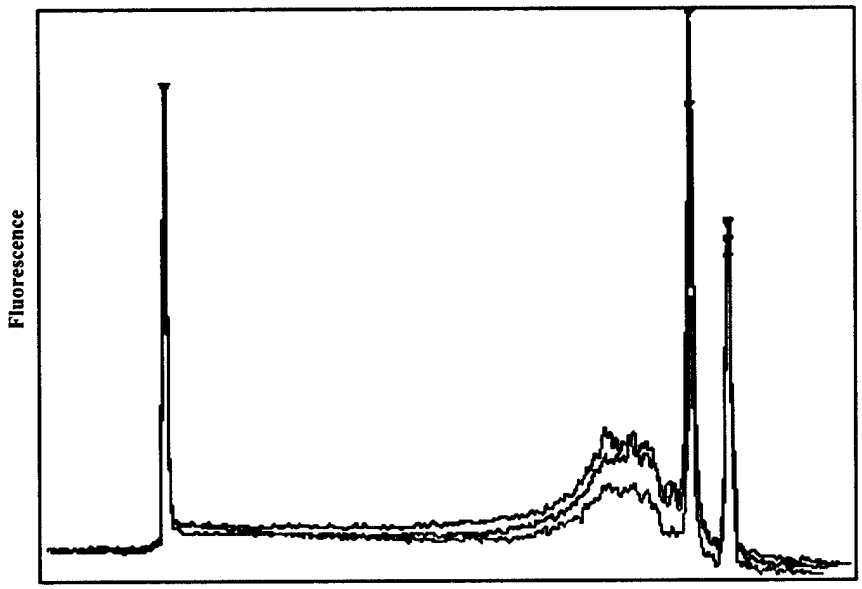

Figure 3. Spectra of three identical VR2516 samples separated on the Bioanalyzer 2100 are shown The fluorescence intensity was measured with respect to migration time of the plasmid in the chip. The first and last peaks (green and red, respectively) corresponded to the lower and upper molecular weight markers respectively. The middle peak (blue) represented the VR2516 plasmid fragment of interest. Areas under the curves may be used to calculate concentration of the plasmid in solution.

Dose Response

The potential to induce protective immunity against infectious *Plasmodium yeolii* sporozoites was tested in a mouse model. A total of five plasmid-based vaccines were tested during this study. The vaccines consisted of three native plasmids and two synthetic plasmid vaccines specifically designed for use in mammals. Vaccines were developed to induce antibodies to proteins present at the surface of infected hepatocytes. In addition to the encapsulation of each vaccine within PLGA particles, the Phase I study initiated work to immunize mice with pDNA/PLGA-based vaccines.

There was no significant T-cell response in mice immunized via a subcutaneous injection of PLGA particles containing the vaccines. Results of the IFN-γ assay for the VR2515/PLGA and naked VR2515 vaccines are presented in Figure 4. Control mice demonstrated a typical immune response to IM administration of naked VR2515. During the course of this study, mice immunized with the vaccine and PLGA particles were not studied in parallel with control animals receiving a subcutaneous injection of the vaccine. Doolan et al. (1996) demonstrated that optimal T-cell response was observed with vaccines administered via an IM route. Subcutaneous injection for the administration of vaccine loaded PLGA microparticles was selected due to the relatively large needle size (20 gauge) required for injection of the particles. Although use of larger needles made IM immunization difficult in the mouse model, it was not expected to be problematic for administration to primates or humans.

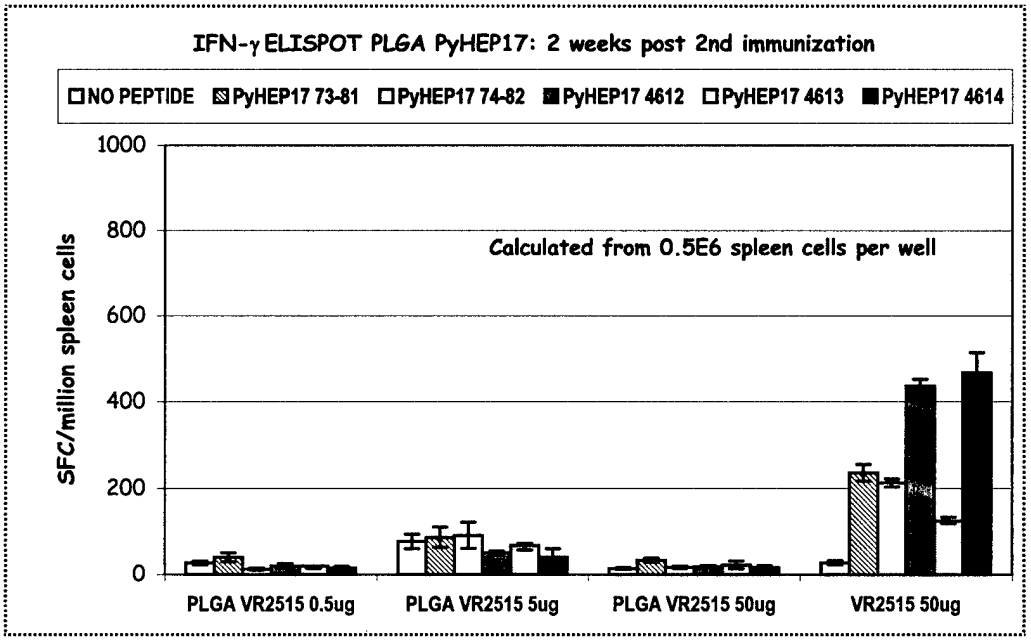

Figure 4. IFN-γ response in mice immunized with VR2515/PLGA and naked VR2515 vaccines was measured. Mice received the control injection of VR2515 consisting of an IM injection of naked pDNA in the tibialis muscle. Plasmid/PLGA vaccines were administered by a subcutaneous injection. There was no significant immune response in mice administered the subcutaneous plasmid/PLGA immunization. Additional studies were proposed to study the immune response of plasmid/PLGA vaccines administered via an IM injection.

4. RECOMMENDATIONS FOR PHASE II

Control of Vaccine Delivery Rate

The administration of a plasmid-based vaccine delivered in a PLGA vehicle will be optimized *in vitro* prior to animal testing in mice. Plasmid/PLGA formulations will be optimized for encapsulation efficiency and the rate at which the pDNA is released from the polymer particles. Control of the plasmid release will be tuned by the loading of the plasmid within the polymer. At reduced loading concentrations, less of the vaccine is available at the polymer surface, alleviating problems involving the immediate loss of DNA upon exposure to aqueous media. Thus, more of the plasmid is retained within the polymer particles and the improved encapsulation efficiency reduces vaccine loss. In addition, loading within the polymer affects diffusion of the pDNA through the matrix. Control of encapsulation and release of the plasmid promotes protection of the plasmid within the polymer matrix from degradation enzymes and extends the period of time over which the pDNA is delivered. During Phase II, plasmid loading will be varied at concentrations between 0.1 and 1 weight percent in the polymer vehicle. Encapsulation efficiency and the rate of plasmid release will be monitored *in vitro* by exposing the pDNA/PLGA particles to phosphate buffered saline (PBS) and incubating at 37°C.

Measurement of Plasmid Concentration

In Phase II, quantification of pDNA released from PLGA matrices can be conducted using the Bioanalyzer. Preliminary studies conducted during Phase I indicate that the Bioanalyzer may be used to measure plasmid in PBS concentrations between 0.5 and 5 ng/mL.

The technique may be used to quantify the amount of plasmid released from PLGA particles using less sample volume than UV spectroscopy. In addition, the technique may be useful in resolving differences in solutions that are relatively close in concentration.

Efficacy of Plasmid/PLGA Vaccines against Malaria

Subcutaneous injection of the pDNA/PLGA-based vaccines failed to invoke an immune response similar to IM administration of naked pDNA. Because previous studies have demonstrated that IM or intradermal (ID) administration optimizes the immune response for the vaccines under study, pDNA/PLGA would ideally be administrated similarly. The experimental plan for Phase II studies include parallel IM administration of naked pDNA and pDNA/PLGA vaccines. Administration of the pDNA/PLGA vaccines would then require IM injections of suspended PLGA particles. Due to potential problems arising with the relatively large needle sizes required to inject PLGA particles and relatively small muscle mass available in the mouse model, an alternative administration approach may be necessary. A potential alternative involves injection of leachate from the particles into the tibialis muscle. Using this procedure, pDNA/PLGA particles would be exposed to saline and the leachate minus the particles would be injected. The schedule of administration of the leachate would be determined by results of the *in vitro* studies. Leachate injection would attempt to simulate the controlled release of the plasmid from PLGA by multiple injections of leachate.

Following successful administration of vaccines in a mouse model, Phase II studies aim to scale the vaccine delivery to primates. The pDNA/PLGA vaccine system will be tested and challenged in primates. Malaria-naïve rhesus monkeys will be randomized into groups of three for each vaccine/PLGA formulation based upon the procedure outlined by Wang et al. (1998). Control groups will receive injections of naked pDNA in saline or an empty PLGA formulation. Immunizations will be conducted at 0, 4, and 8 weeks via administration of pDNA/PLGA suspensions. Injections will consist of 1 mL total volume delivered IM amongst four sites: triceps, tibialis anterior, deltoid, and quadriceps. Blood samples will be collected at two and four weeks post-immunization corresponding to weeks 6, 8, 10 and 12.

Blood samples will be tested for antibodies and T-cell response using techniques outlined by Wang et al. (1998). As in the mice study, antibody levels will be monitored by IFAT tests where sera are exposed to air-dried sporozoites as described previously. CTL activity will be determined by *in vitro* restimulation of peripheral blood mononuclear cells (PMBCs) incubated with target cells containing the antigen of interest. Net percent specific cell lysis will be calculated based upon results from a release assay with the ^{51}Cr-labeled target cells. Finally, IFN-γ response will be measured by detection of IFN-γ mRNA levels. Briefly, PBMCs are stimulated in the presence of infected recombinant viruses for 8 h and total RNA is extracted. IFN-γ mRNA probes are constructed by PCR techniques and mRNA levels are measured by densitometric analysis.

Rhesus monkeys immunized with pDNA/PLGA or naked pDNA vaccines will be malaria-challenged to determine vaccine efficacy. The monkeys will be challenged four weeks post the third immunization (Week 12) by a controlled intravenous injection with infectious sporozoites. The response of challenged monkeys for malaria (positive or negative) will be correlated with the previously determined antibody levels, CTL activity, and vaccine type.

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